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The Development of Skeletal Muscle in Young Horses: An Ultrasonography and Satellite Cell Approach

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The Development of Skeletal Muscle in Young Horses: An Ultrasonography and Satellite Cell Approach

Emma LaVigne

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2015

Abstract

Muscle growth in young horses is characterized by an increase in muscle cross-sectional area, which can be accomplished through the activation and differentiation of satellite cells. Satellite cells can be stimulated or inhibited in response to different cytokines and growth factors and are key mediators of muscle hypertrophy and regeneration. The aims of this study were to 1) investigate the growth of the longissimus dorsi (LD) muscle in horses under 5 years of age and, 2) to determine the effects of cytokines and growth factors on satellite cell behavior in foals. The area, width, height, and subcutaneous fat depth were measured using ultrasonography at 6-month increments over the course of 1 year in 14 horses from the University of Connecticut herd. Satellite cells obtained from 10 day old foals ($n = 4$) were cultured in the presence of the cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)- α , and the growth factors insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF)-2 to determine the effects on proliferation and differentiation. Data were analyzed using the MIXED procedure in SAS. Longissimus dorsi area, height, and height to width ratio were significantly increased by time and age of horse ($P < 0.02$), although there was no interaction of time and age. Interestingly, there was a significant increase in LD area from April to October, but not from October to April. Interleukin-6 decreased proliferation of satellite cells by 14.9% ($P = 0.01$) and TNF- α decreased proliferation of satellite cells by 11.5% ($P = 0.001$). Interleukin-6 increased fusion by 6.2% ($P = 0.001$) and TNF- α decreased differentiation of satellite cells by 8.7% ($P < 0.0001$) compared with control cells. Satellite cell proliferation was increased by 28.8% in the presence of IGF-1 ($P < 0.0001$) and by 73.0% in the presence of FGF-2 ($P < 0.0001$). Insulin-like growth factor-1 and FGF-2 were also different from each other ($P < 0.0001$). Differentiation was decreased in the presence of FGF-2 (13.1%, $P < 0.0001$) and increased in the presence of IGF-1 (3.5%, $P =$

0.0087). The LD undergoes notable growth over the course of a year in weanlings to five-year-old horses with the most significant growth occurring from Apr to Oct. By differentially stimulating or inhibiting proliferation and differentiation of satellite cells, IL-6, TNF- α , IGF-1 and FGF-2 significantly affect muscle hypertrophy and regeneration. Dysregulation of these cytokines or growth factors, therefore, can lead to detrimental muscle degradation, inflammation, and impaired growth.

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Introduction

Proper muscle growth is critical to the health and performance of all types and breeds of horses from the pleasure horse to the racehorse. Muscle provides the power necessary for racehorses, show jumpers, and performance horses alike to reach their maximum agility levels and speed. Recognizing the various factors that affect muscle growth can lead to better health practices to maximize a horse's athletic capabilities. Prenatal muscle growth is characterized by an increase in the number of muscle fibers. Postnatal growth, however, is mainly a result of fiber hypertrophy due to increased muscle protein accretion, specifically the contractile proteins. The mechanisms by which muscle changes are complex and involve various signaling molecules and pathways. The inflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)- α , as well as the growth factors insulin-like growth factor (IGF)-1 and fibroblast growth factor (FGF)-2, are critical mediators of skeletal muscle (Li, 2003; Nielsen et al., 2007). Muscle fiber growth and repair are accomplished by activation of satellite cells, or muscle stem cells. Satellite cells mediate muscle hypertrophy as well as maintain the myonuclear domain (Chargé and Rudnicki, 2003). A thorough understanding of cytokine and growth factor signaling is critical to optimize muscle growth and repair, and to ultimately enhance athletic performance.

Muscle in the Growing Animal

As horses and other large domestic animals age, their muscle composition and enzyme expression change considerably. From the limited amount of information known about horse muscle development, it is possible to make several conclusions. Horses, like many other mammals, experience an increase in overall muscle size as they age. Due to the limited data available on horse muscle, it is appropriate to use cattle as a model to provide information on the

basic growth and development of muscle in large animals. In a study conducted by Albrecht et al. (2006), four breeds of beef cattle were slaughtered at 2, 4, 6, 12, and 24 months and muscle samples were collected from the semitendinosus muscle to measure differences in muscle fibers. It was concluded that as the cattle grew, there was a significant increase in the cross-sectional area of the muscle fibers, bundles, and muscles as a whole. Different breeds of cattle tended to grow differently. For example, the Belgian Blue showed a five-fold increase in the muscle cross-sectional area versus a four-fold increase in other breeds from 2 to 24 months of age. In all animals studied thus far, post-natal muscle development is mainly a result of hypertrophy as opposed to hyperplasia since the number of muscle fibers is fixed before birth. It is reasonable to conclude that the genetic makeup of an animal, as well as breed, significantly influences muscle fiber type and number.

In response to early training in growing horses, several changes in the muscle occur. According to Rietbroek et al. (2007), the cross-sectional area of all fiber types increased in young trained horses, and the proportion of oxidative muscle fibers increased, particularly the intermediate fiber type IIa. There was a decrease in the proportion of glycolytic fibers, especially the intermediate fiber type IIad. On the molecular level, there was an increase in citrate synthase activity, which is an enzyme involved in the conversion of acetyl CoA and oxaloacetate to coenzyme A and citrate respectively in the citric acid cycle. This increase indicated a higher oxidative capacity. There was also a decrease in Na^+, K^+ - ATPase activity, which maintains excitability and force in a muscle by means of creating an action potential. This indicated that there was a decrease in the surface area to volume ratio as the horses developed.

As horses continue to age, the muscle decreases in oxidative capacity and begins to undergo degeneration. Kim et al. (2005) reported that there was no significant relationship

between myosin heavy chain isoforms and age, but citrate synthase activity decreased and lactate dehydrogenase activity increased. Both of these parameters suggest a decrease in the oxidative capacity of muscle and an increase in lactate production through glycolysis. This indicates that aerobic energy pathways are not as efficient and glycolytic pathways are induced, which would suggest an increase in the proportion of glycolytic fibers and a decrease in the proportion of oxidative fibers. The timeline of events from muscle development to maintenance, to degeneration has yet to be investigated.

The Use of Ultrasound to Monitor Muscle Development

Ultrasound is a non-invasive technique that is useful and accurate for measuring muscle cross-sectional area. According to Pillen (2010), it is important to relate what is seen on the ultrasound screen with the anatomy of the animal and to identify when something is abnormal. Muscle tissue on an ultrasound appears black because of its high water composition, but white spots may be visualized due to connective tissue between muscles and muscle groups since it is relatively dense. Adipose tissue also appears dark on the screen. Because ultrasound images are often difficult to interpret, it is critical for the operator to have a solid background in anatomy. With ultrasound images, the area of a muscle or muscle group can be determined. This is extremely useful in investigative studies since it is possible to put an objective measurement to muscle atrophy and hypertrophy, and determine how the area changes over time or in response to different treatments. Ultrasound is also useful in diagnosing neuromuscular disorders. Healthy muscle has low echo intensity and appears dark on the screen. When muscle increases in echo intensity, it appears lighter in the image due to an increased concentration of fat and fibrous connective tissue that should not normally be present.

The use of ultrasound can be quite subjective, depending on the investigator. Herring et al. (1994) evaluated the accuracy and repeatability of ultrasound measurement technique. Three technicians using two different machines measured the longissimus dorsi muscle area and back fat in steers. Steers were then slaughtered to compare these measurements to the actual carcass composition. Differences existed between machines and between technicians, and measurements deviated slightly from the actual carcass measurements. Overall, however, the interpretations of ultrasound measurements were comparable to direct carcass measurements. By minimizing as many variables as possible and keeping the ultrasound personnel, equipment and the settings of the machine constant, ultrasound measurement was found to be both accurate and repeatable.

Lindner et al. (2010) also investigated repeatability and reproducibility of ultrasound measurements in horses of the extensor carpi radialis, the extensor digitorum longus, the gluteus medius, the longissimus lumborum, the semitendinosus and the supraspinatus muscles. The examiners did have an effect on the mean coefficient of variance (CV) when measuring the extensor carpi radialis and the extensor digitorum longus. The CV was calculated at around 10-20% for the flexed extensor digitorum longus and the supraspinatus and was around 3-7% for the extended extensor carpi radialis and longissimus lumborum, indicating the least and most agreement respectively. When only one examiner took measurements of the longissimus dorsi muscle as described by Abe et al. (2012), the test and re-test measurements varied only by 1-2% and were highly reproducible. When compared to the wide variance of test and re-test measurements by each examiner and between each examiner described in Lindner et al. (2010), the collective information suggests that ultrasound is highly repeatable and reproducible, but one trained examiner may provide the best results. Sources of variability could include confidence

with the ultrasound machine and horse, position of the horse's head and body, and image clarity of the ultrasound (Lindner et al., 2010; Abe et al., 2012).

An Introduction to Muscle Physiology and Satellite Cells

The structure and composition of muscle tissue allows it to respond quickly to external and internal stimuli including stretch, and injury. Muscle is composed of individual cells called myofibers, which are created by the fusion of myoblasts during growth and development (Chargé and Rudnicki, 2003). Myofibers are multinucleated and have peripherally located nuclei to allow space for sarcomeres, the units of contractile proteins. Individual myofibers are made up of myofibrils, which are composed of actin and myosin filaments as well as troponin and tropomyosin. When these proteins interact, they are responsible for contraction of the muscle. Degradation of any of these proteins can lead to muscle wasting.

Skeletal muscle hypertrophy is mediated by an increase in protein synthesis (Yablonka-Reuveni and Rivera, 1994). Increased synthesis of contractile proteins such as actin and myosin increases the number of sarcomeres and results in a larger fiber cross-sectional area. Satellite cells also contribute to hypertrophy through the addition of nuclei as well as proteins, mitochondria, and other organelles to the fiber. Satellite cells are located on the periphery of the fiber between the sarcolemma and the basal lamina and are activated during growth and repair. The number of muscle fibers is fixed at birth; therefore, protein synthesis and satellite cell activation are the major mechanisms for muscle growth. Satellite cells contribute myonuclei to the muscle fibers to maintain a constant number of nuclei in relation to the amount of cytoplasm, referred to as the myonuclear domain (Chargé and Rudnicki, 2003). Maintenance of the myonuclear domain is important since the nucleus houses the necessary genetic material for

synthesizing contractile proteins and maintaining regulatory control of the fiber. When a fiber is damaged, satellite cells are activated along the entire length of the fiber. Failure of satellite cells to incorporate into the fiber results in impaired muscle regeneration.

Several different transcription factors regulate myoblast proliferation and differentiation. According to Chargé and Rudnicki (2003), both quiescent and proliferating satellite cells express a regulatory transcription factor called paired box protein 7 (Pax7). When satellite cells are signaled to differentiate, expression of Pax7 is downregulated whereas expression of myogenic determination protein (MyoD) and myogenic factor 5 (Myf5) are upregulated. The regulatory factors MyoD and Myf5 are responsible for committing the cells to the myogenic lineage. At this stage, Pax7 expression increases in some of the activated satellite cells, which return to the quiescent state to replenish the pool of satellite cells. In the remainder of the activated satellite cells, increased expression of myogenin causes the cells to exit the cell cycle and begin to fuse into myofibers. Finally, myogenic regulatory factor (MRF) 4 initiates the formation of contractile proteins present in mature fibers.

Effects of FGF-2 and IGF-1 on Satellite Cells

Satellite cells are activated by a multitude of different factors including stretch, damage due to physical activity, fiber hypertrophy, and production of nitric oxide (Yablonka-Reuveni et al., 1994). Greene et al. (1991) reported that several growth factors affect satellite cell proliferation and/or differentiation. Insulin-like growth factor (IGF)-1 is a protein growth factor that plays a major role in the somatotrophic axis. It is released from the liver by growth hormone (GH) produced in the anterior pituitary gland to act on muscle cells in an endocrine manner, or it is produced in an autocrine/paracrine manner from the muscle cells themselves to stimulate

myoblasts to proliferate. Fibroblast growth factor (FGF)-2 is produced by fibroblasts as well as muscle cells, and generally increases proliferation in muscle cells but has an inhibitory effect on differentiation. In an investigation conducted on bovine satellite cells *in vitro*, dose response curves were created for both IGF-1 and FGF-2. Fibroblast growth factor-2 increased proliferation in a dose-dependent manner, but decreased differentiation at concentrations above 3 ng/mL. Insulin-like growth factor-1 did not significantly increase proliferation, but a notable increase in differentiation was observed. When cells were treated with FGF-2 and IGF-1 together, high concentrations of FGF-2 enabled IGF-1 to increase proliferation. In satellite cells isolated from adult horses, FGF-2 was more effective at increasing proliferation than IGF-1 as determined by methylene blue stain incorporation (Byrne et al, 1998).

Allen and Boxhorn (1989) reported that in satellite cells isolated from rats, IGF-1 increased proliferation in a dose-dependent manner ranging from 0.1ng/mL to 10ng/mL, even in the presence of transforming growth factor (TGF)- β , a strong inhibitor of differentiation. Insulin-like growth factor-1 also stimulated a significant dose-dependent increase in differentiation, but only in the absence of TGF- β . Fibroblast growth factor-2 strongly stimulated myoblast proliferation, but inhibited differentiation. The largest increase in proliferation, differentiation and fusion occurred in the presence of both IGF-1 and FGF-2, indicating a synergistic effect of these two growth factors. However, little is known about the affect of these growth factors on equine satellite cells.

Inflammatory Cytokines IL-6 and TNF- α

Interleukin-6 and TNF- α are commonly considered pro-inflammatory cytokines, as is IL-1, which is predominantly involved in the immune response. These cytokines are necessary for

stimulating muscle repair following strain and exercise (Li, 2003; Serrano et al., 2008; Begue et al., 2013; Podbregar et al., 2013). Following injury, macrophages accumulate in the muscle and produce a significant amount of TNF- α and IL-1, which are upstream regulators of IL-6.

Interleukin-6 and TNF- α are also released directly from muscle and are therefore identified as myokines. Muñoz-Cánoves et al. (2013) reported that cytokines of the IL-6 family commonly use the glycoprotein (gp)130 receptor or the IL-6 receptor α (IL-6R α). Signaling is mediated by either the classical or the trans-signaling mechanism. In the classical pathway, IL-6 binds to IL-6R, which associates with a homodimer of gp130 to initiate JAK/STAT signal transduction. Liver cells and immune cells such as macrophages, lymphocytes and neutrophils typically have this transmembrane receptor. The trans-signaling mechanism, however, allows cells that do not have transmembrane IL-6R, such as muscle cells, to respond to IL-6. Proteolytic processing of IL-6R causes secretion of an extracellular soluble receptor, sIL-6R, that can bind to IL-6 as well as the gp130 homodimer present on many cell types to activate the JAK/STAT signal transduction pathway.

Interleukin-6 is released in response to resistance exercise. Nielsen et al. (2007) stated that circulating IL-6 concentrations, the rate of transcription of the IL-6 gene, and the synthesis of the IL-6 receptor increase immediately following exercise and decrease thereafter. The concentration is related to the intensity and the duration of the activity and the physical fitness of the individual performing the activity. An increased number of receptors allows for enhanced sensitization of the muscle tissue to the binding of IL-6. Interleukin-6 is released into the circulation, where it can act as a hormone to release other anti-inflammatory cytokines as well as suppress the effects of TNF- α on insulin resistance. Interleukin-6 may also have a role in endogenous glucose production through the action of an unidentified factor on the liver. It also

positively influences insulin sensitivity in the peripheral tissues. Infusion of IL-6 in healthy humans causes lipolytic effects, indicating that this cytokine may affect lipolysis and fat oxidation. Interleukin-6 knockout mice, or mice with a mutated and inoperative IL-6 gene, developed late-onset obesity and impaired glucose tolerance. This suggests that a lack of IL-6 results in decreased glucose uptake, most likely due to decreased insulin sensitivity. Washington et al. (2011) stated that IL-6 also stimulates the release of insulin-like growth factor (IGF)-1, which is an important growth factor that signals muscle cell proliferation and differentiation. The primary function of IL-6, however, is to stimulate inflammation that signals muscle repair.

Tumor necrosis factor- α is an inflammatory cytokine that is released from both immune cells and myocytes (Li, 2003). Tumor necrosis factor- α mediates inflammatory, cytotoxic, and apoptotic responses in muscle to cause catabolism and degradation of muscle proteins. Much like IL-6, TNF- α modulates growth and differentiation in certain cell types, including muscle. Tumor necrosis factor- α and its receptors affect the growth and development of prenatal and neonatal animals. Repeated injections of TNF- α antibodies in pregnant mice resulted in growth retardation of the fetus, indicating inadequate proliferation and differentiation of myoblasts. Mice deficient in TNF- α receptor-associated factor 2, a signaling molecule required for TNF- α receptor activation, are born with significantly smaller muscle mass. Additionally, a double knockout of the TNF- α receptor impaired strength recovery in freeze-induced injury in adult mice. In accordance with these statements, Podbregar et al. (2013) confirmed that TNF- α also contributes to IL-6 regulation and, therefore, both directly and indirectly stimulates muscle growth and repair. During muscle regeneration, TNF- α , IL-1 and their receptors are increased. Thus, TNF- α can promote muscle regeneration directly, as well as stimulate IL-6 to increase proliferation and differentiation of myoblasts. Tumor necrosis factor- α also contributes to insulin resistance and

protein degradation at very high physiological concentrations. It can be inferred from these findings that TNF- α contributes to muscle growth, but its release may also be a protective mechanism to stimulate muscle repair in response to damage and exercise.

Effects of IL-6 and TNF- α on Satellite Cells

Stimulation of mitotic activity and differentiation of satellite cells and myoblasts is mediated by the secretion of IL-6 and TNF- α (Li, 2003, Serrano et al., 2008). Serrano et al. (2008) reported that in IL-6 knockout mice, there were fewer MyoD expressing satellite cells and fewer proliferative cells, indicating impaired proliferation and differentiation. Interleukin-6-deficient myoblasts in vitro also had reduced content of MyoD. Recovery of muscle mass following atrophy is associated with an increase in IL-6 expression (Washington et al., 2011). However, in IL-6 knockout mice, expression of the IGF-1 gene was reduced by 80% during hind limb suspension, and was much slower to increase during reloading and recovery. After the first day of recovery, MyoD and myogenin mRNA expression increased 3-fold and 2-fold, respectively, in wild type mice, but mRNA expression was not induced in IL-6 knockout mice. The results imply that IL-6 is imperative for proliferation and differentiation of satellite cells and myoblasts, and that a lack of IL-6 in muscle cells can significantly impede these processes.

Interleukin-6 is also responsible for stimulating satellite cell incorporation into myofibers following proliferation and differentiation into myoblasts. Serrano et al. (2008) reported that myonuclei were increased by 40% in response to overload in wild-type mice, whereas no increase was observed in IL-6 knockout mice. This indicates failure of satellite cells to proliferate and differentiate in the absence of IL-6. Migration of satellite cells to establish contact with cell fibers was also significantly reduced in IL-6 knockout mice, but replacement with

recombinant IL-6 attenuated this effect. Similarly, Washington et al. (2011) reported that the gastrocnemius muscle weight in IL-6 knockout mice was also decreased compared with wild-type mice during recovery from hind limb suspension. This delay in recovery was most likely due to impaired IGF-1 mRNA expression since IGF-1 expression was not detected in IL-6 knockout mice after one day of recovery. Sustained increased concentrations of IL-6 caused inflammation and inhibition of muscle regeneration. Myoblasts treated with IL-6 for 48 hours showed decreased fusion, decreased expression of myogenic regulatory factors and inhibited IGF-I signaling (Al-Shanti and Stewart, 2012). Greater concentrations or long-term treatment with IL-6 also increased proteolysis in murine myotubes, characteristic of muscle atrophy. Furthermore, blocking IL-6 signaling with an IL-6R antibody reduced muscle atrophy (Muñoz-Cánoves et al., 2013).

Tumor necrosis factor- α has a similar effect on proliferation and differentiation of satellite cells and myoblasts. In an experiment conducted by Li (2003), incubation of pre-myoblasts with TNF- α caused a dose-dependent increase in DNA content, indicating that it is responsible for stimulating DNA synthesis. An average of six proliferative satellite cells were present in muscle sections of mice injected with TNF- α , whereas no proliferative cells were identified in control groups. Li (2003) also determined that TNF- α concentrations increased within 5 hours after exercise and peaked at around 24 hours, which parallels the time when satellite cells are activated. Podbregar et al. (2013) stated that the action of TNF- α is more prominent in proliferating myoblasts, but still has limited effects on myotubes. This could be a protective mechanism to reduce pro-inflammatory cytokines once differentiation has been initiated.

The activity of TNF- α differs depending on the stage of differentiation and the physiological state of the individual (Podbregar et al., 2013). Magee et al. (2012) reported that although TNF- α concentrations are increased transiently in the regeneration process, greater concentrations for a prolonged period of time results in inflammation and muscle degradation. In murine satellite cells treated with TNF- α , myotube fusion was decreased, confirmed by a 40% decrease in myogenic fusion index and 50% decrease in myotube width. According to Degens (2010), TNF- α has a more prominent effect on muscle degradation in the aged population. This is due to chronic inflammation in the skeletal muscle and accumulation of damaged proteins that result from toxic substances like nitric oxide and free radicals. TNF- α activates NF- κ B, which subsequently activates the ubiquitin-proteasome pathway that degrades MyoD and myogenin proteins. Therefore, satellite cells are able to proliferate in the presence of TNF- α , but differentiation is impaired. Tumor necrosis factor- α may also cause selective apoptosis of satellite cells. Low-grade inflammation and expression of TNF- α is imperative for the normal repair of muscle tissue via activation of satellite cells, but during the aging process as well as in response to certain myopathies, chronic inflammation and exposure to TNF- α and other pro-inflammatory cytokines causes muscle protein degradation and impaired recruitment of satellite cells.

Conclusions

Much research is still needed to determine how muscle develops throughout a horse's life and what can be done in terms of training and management to optimize their health. Most of the changes that occur post-natally in young horse muscle are associated with hypertrophy of muscle fibers as well as a gradual shift in muscle type from glycolytic to oxidative fibers as horses

develop (Rietbroek et al., 2007). Satellite cells are key mediators of muscle hypertrophy and repair. They contribute myonuclei to the growing myofiber to maintain adequate amounts of protein synthesis to support the increase in cross-sectional area of the fiber (Chargé and Rudnicki, 2003). Insulin-like growth factor-1 and FGF-2 are well characterized as key mediators of muscle growth, where IGF-1 stimulates both proliferation and differentiation and FGF-2 stimulates proliferation, but can inhibit differentiation (Greene et al., 1991; Byrne et al, 1998; Allen and Boxhorn, 1989). Interleukin-6 and TNF- α are involved in mediating the inflammatory response in myofibers after exercise (Li, 2003; Nielsen et al., 2007; Podbregar et al., 2013). This response is essential for the stimulation of muscle repair and regeneration. Large or unregulated physiological concentrations of these inflammatory cytokines in diseased states can cause severe muscle damage (Li, 2003; Nielsen et al., 2007; Serrano et al., 2008; Podbregar et al., 2013). More research is necessary to determine the physiological conditions and concentrations at which IL-6 and TNF- α influence muscle repair as opposed to muscle damage. Controlled and coordinated secretion of IL-6 and TNF- α to stimulate satellite cell activity decreases chances of injury and enhances muscle growth, so that athletes can perform at their greatest potential. Thus, the objectives of this work are to 1) to measure changes in the longissimus dorsi muscle in young horses over the course of one year, and 2) to determine how IL-6, TNF α , FGF-2 and IGF-1 affect proliferation and differentiation of satellite cells from foals. Specifically, we hypothesize that:

1. The cross-sectional area of the longissimus dorsi as well as fat depth, muscle height, and muscle width will increase throughout the course of a year. The ratio of height to width will also increase.
2. IGF-1 will increase proliferation and differentiation of equine satellite cells.
3. FGF-2 will increase proliferation, but decrease differentiation of equine satellite cells.

4. IL-6 and TNF- α will increase proliferation but decrease differentiation of equine satellite cells.

Materials and Methods

All procedures were approved by the University of Connecticut IACUC.

Muscle Ultrasonography

Ultrasound images of the longissimus dorsi (LD) muscle between the 13th and 14th rib were obtained from 14 horses at the University of Connecticut age 6 months to less than 5 years. Horses were divided into three groups based on age: Weanlings (n = 5), yearlings and 2-year-olds (n = 4), and 3 and 4-year-olds (n = 5). An Aloka 500V Ultrasound Machine with a 3.5 MHz transducer was used to obtain images (Corometrics Medical Systems, Inc., Wallingford, CT). Images were taken at three different time points over the course of a year. The first time point was in October 2013, the second was in April 2014, and the third was in October 2014. Three independent examiners measured the cross-sectional area, height, and width of the LD and the subcutaneous fat depth using AUSKey for Windows 5.10 (Animal Ultrasound Services, Inc., Ithaca, NY).

Muscle Biopsy

Samples from the semitendinosus of 10-day-old foals (n = 4) from the University of Connecticut's herd were collected in 2013. A 3 cm incision was made in the skin at the midpoint of the semitendinosus to expose the muscle. A 1-2 g biopsy was removed from the midpoint of the muscle. The incision was closed with sutures and skin glue. The muscle sample was placed in

phosphate buffered saline (PBS) and transported to the lab for satellite cell isolation following standard procedures. Satellite cells were cryopreserved until further use.

Satellite Cell Culture

Satellite cells were seeded on 10 cm plates at 1.8×10^4 cells/cm² and cultured in growth media [20% Fetal Bovine Serum (FBS), 0.2% gentamicin, 0.3% Fungizone, 1% penicillin/streptomycin, and 4 ng/mL FGF-2 in high glucose Dulbecco's Modified Eagle Medium (DMEM)]. Media was changed every 48 hours. Cells were passaged at 60-70% confluence. For proliferation assays, cells were seeded at 2.6×10^3 cells/cm² on 12 well culture plates in triplicate wells for each treatment. After 24 hours in culture, the media was changed to 2% FBS in DMEM and supplemented with 10 ng/mL IL-6, 20 ng/mL TNF- α , 25 ng/mL IGF-1, 10 ng/mL FGF-2, or carrier only (control). All cytokines and growth factors were purchased from R&D Systems. Cells were allowed to proliferate for 24 hours in treatment media before pulsing with 10 μ M EdU for 30 minutes (Alter et al., 2008, Greene et al., 1995, Villalta et al., 2011). Cells were fixed in 4% paraformaldehyde for 15 minutes and immunostained for EdU according to the manufacturer's protocol (Life Technologies, Eugene, OR) to identify cells in the S phase of mitosis. Hoescht dye (Life Technologies, Eugene, OR) was used to identify all nuclei.

For differentiation assays, cells were seeded at 7.5×10^4 cells/well on 12-well plates in 20% FBS growth media in triplicate wells for each treatment and cultured for 96 hours when cells were 100% confluent. The media was changed to differentiation media (2% FBS in low glucose DMEM) supplemented with 10 ng/mL IL-6, 20 ng/mL TNF- α , 25 ng/mL IGF-1, 10 ng/mL FGF-2, or carrier only (control). Cells were allowed to differentiate for 48 hours before being fixed in 4% paraformaldehyde for 15 minutes and immunostained for myosin heavy chain

(MyHC) expression using MF-20 supernatant as the primary antibody (Developmental Studies Hybridoma Bank) and AlexaFluor 488 goat anti-mouse secondary fluorescent antibody (Invitrogen Molecular Probes, Eugene, OR). Hoescht 33342 dye (Invitrogen Molecular Probes, Eugene, OR) was used to identify nuclei. The fusion index was calculated as the number of nuclei present in multi-nucleated MyHC-positive myofibers divided by the total number of nuclei.

Microscopy

Fluorescent images were obtained with either a Nikon TiE microscope with Intensilight Illuminator used with either an Andor iXon 897 EM-CCD camera or an Andor Clara megapixel CCD camera or a Zeiss Axiovert 200 with a Sutter DG4 illuminator and Hamamatsu Orca-ER CCD camera. μ Manager software funded by the National Institute of Health (NIH) was used for imaging (Edelstein et al., 2010). Image J software (NIH) was used for counting cell nuclei. Six images were taken per well for proliferation assays and eight images were taken per well for differentiation assays.

Statistics

Ultrasound data were analyzed by the MIXED procedure in Statistical Analysis Software (SAS). Satellite cell proliferation and differentiation data were also analyzed using the MIXED procedure in SAS. Data are presented as mean \pm SEM. $P < 0.05$ was considered significant and $P < 0.10$ was considered a trend.

Results

Changes in the Cross-Sectional Area, Height, and Width of the LD

There was a main effect of age ($P < 0.05$) and date ($P < 0.0001$) on the cross-sectional area of the LD, but there was no interaction (Fig. 1A). The area of the LD was not different from Oct 13 to Apr 14 in Weanlings, Y-2, or 3-4. The area of the LD in Oct 14 was different from the area of the LD in Oct 13 and Apr 14 in Weanlings, Y-2, and 3-4. The area of the LD of Weanlings was different from the area of the LD in 3-4, but not from Y-2 horses. There was no difference in LD area between Y-2 yr olds and 3-4 yr olds.

The ratio of LD height to width in Oct 14 was different from the ratio in Oct 13 and Apr 14 (Fig. 1B, $P < 0.0001$). The ratio in Weanlings was different from that of 3-4 ($P < 0.05$), but it was not different from that of Y-2. The ratio in Y-2 was not different from that of 3-4. There was no interaction of date and age.

There was a main effect of age ($P < 0.05$) and date ($P < 0.0001$) on the height of the LD, but there was no interaction (Fig. 1C). The height of the LD from Oct 13 to Apr 14 was not different in Weanlings, Y-2 or 3-4. The height of the LD in Oct 14 was different from the height of the LD in Oct 13 and Apr 14 in Weanlings, Y-2, and 3-4. The height of the LD of Weanlings was different from the height of the LD in 3-4, but not from Y-2 horses. There was no difference in LD height between Y-2 yr olds and 3-4 yr olds.

There was a main effect of date on the width of the LD ($P < 0.01$), but no effect of age (Fig. 1D). The width of the LD in Oct 14 was different from that in Oct 13 and Apr 14. The width of the LD was not different from Oct 13 to Apr 14.

There were no effects of age or date on subcutaneous fat depth in any group (data are not shown).

Effects of TNF- α and IL-6 on myoblast proliferation and differentiation

Interleukin-6 decreased myoblast proliferation by 14.9% compared with controls (Fig. 2, $P = 0.001$). Proliferation was decreased by 11.5% in myoblasts treated with TNF- α compared with controls ($P = 0.01$).

The fusion index was increased by 6.2% in myotubes treated with IL-6 compared with controls (Fig. 3, $P = 0.001$). Tumor necrosis factor- α decreased fusion index by 8.7% compared with controls ($P < 0.0001$). Satellite cells treated with IL-6 were significantly different from those treated with TNF- α ($P < 0.0001$).

Effects of IGF-1 and FGF-2 on myoblast proliferation and differentiation

Insulin-like growth factor-1 increased the number of Edu-positive satellite cells by 28.8% compared with the control group (Fig. 4, $P < 0.0001$). Fibroblast growth factor-2 increased the number of EdU-positive cells by 73.0% compared with the control ($P < 0.0001$). Proliferation of satellite cells treated with FGF-2 was significantly increased compared with proliferation of cells treated with IGF-1 ($P < 0.0001$).

The fusion index was increased in the presence of IGF-1 by 3.5% compared with the control group ($P = 0.0087$), but fusion index was decreased in the presence of FGF-2 by 13.1% compared with the control group ($P < 0.0001$). Satellite cells treated with IGF-1 were significantly different from those treated with FGF-2 ($P < 0.0001$).

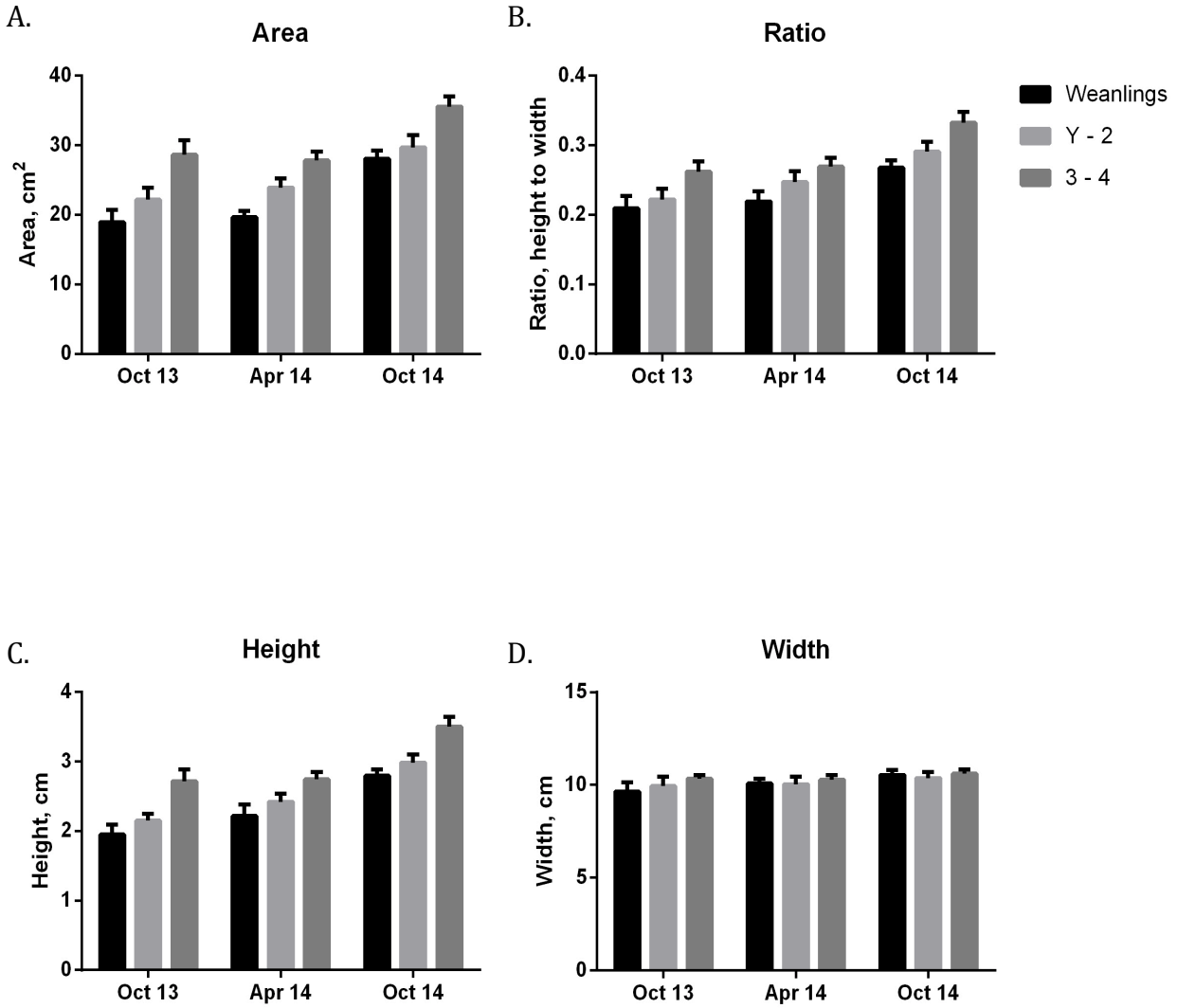


Figure 1. Changes in LD with age. The LD was visualized using ultrasonography and images were analyzed for cross sectional area (CSA), height, width, and height:width ratio. The CSA of the LD increases as horses age and across the span of 1 year (A). The height:width ratio of the LD increases as horses age and across the span of 1 year (B). The height of the LD increases as horses age and across the span of 1 year (C). The width of the LD increases across the span of 1 year (D). Data are presented as mean \pm SEM. $P \leq 0.05$.

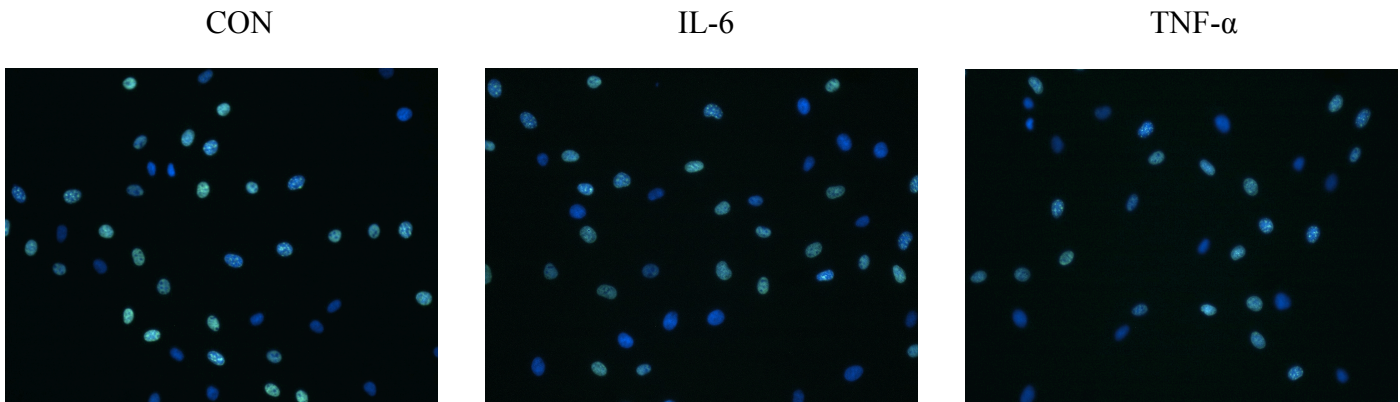
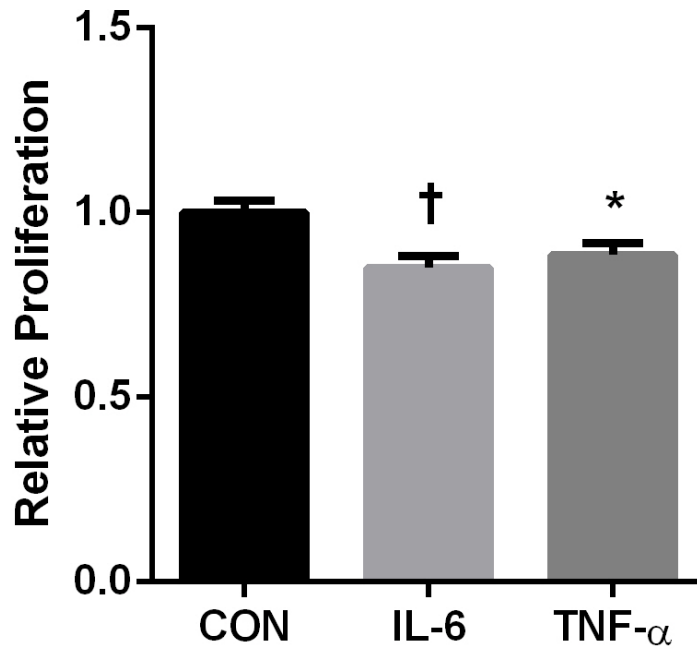
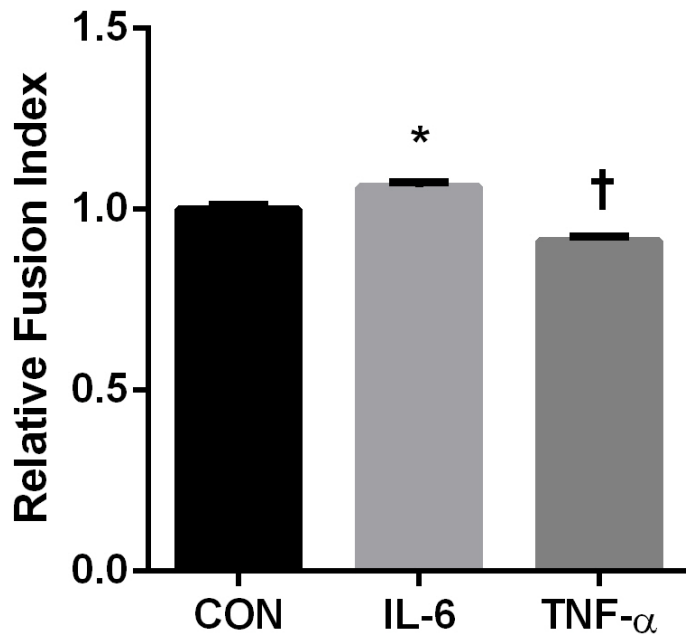


Figure 2. Interleukin-6 and TNF- α decrease equine satellite cell proliferation. Satellite cells were cultured for 24 h in the presence of 10 ng/mL IL-6, 20 ng/mL TNF- α or control media (CON). The number of proliferative cells was determined by EdU incorporation. Interleukin-6 and TNF- α decreased satellite cell proliferation compared with control cells (CON; 14.9% and 11.5%, respectively; $P \leq 0.01$). Data were normalized to controls and are presented as mean \pm SEM. * $P \leq 0.01$, † $P \leq 0.001$ compared with CON; $n = 4$ per treatment. Representative images are shown where blue indicates satellite cell nuclei and green indicates proliferative nuclei positive for EdU.



CON

IL-6

TNF- α

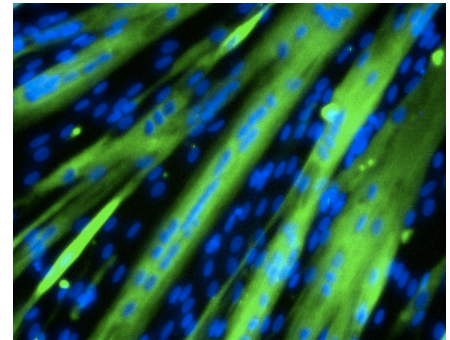
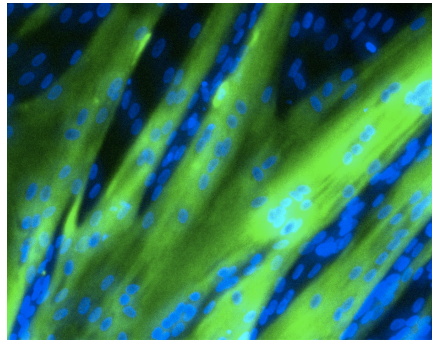
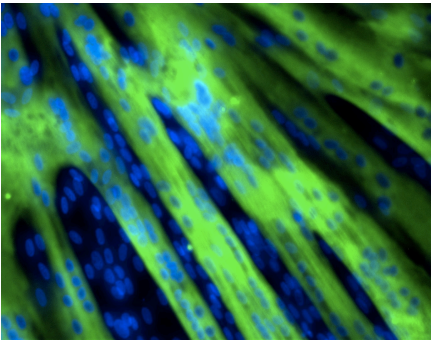


Figure 3. Interleukin-6 increases equine satellite cell differentiation and TNF- α decreases equine satellite cell differentiation. Satellite cells were cultured for 48 h in the presence of 10 ng/mL IL-6, 20 ng/mL TNF- α or control media (CON). Relative differentiation was determined by the fusion index. Fusion into myotubes was increased 6.2% in the presence of IL-6 ($P = 0.001$) but decreased 8.7% by TNF- α ($P < 0.0001$) compared with controls (CON). Data were normalized to controls and are presented as mean \pm SEM. * $P \leq 0.001$, † $P < 0.0001$ compared with CON; $n = 4$ per treatment. Representative images are shown where blue indicates satellite cell nuclei and green indicates myofibers positive for MyHC.

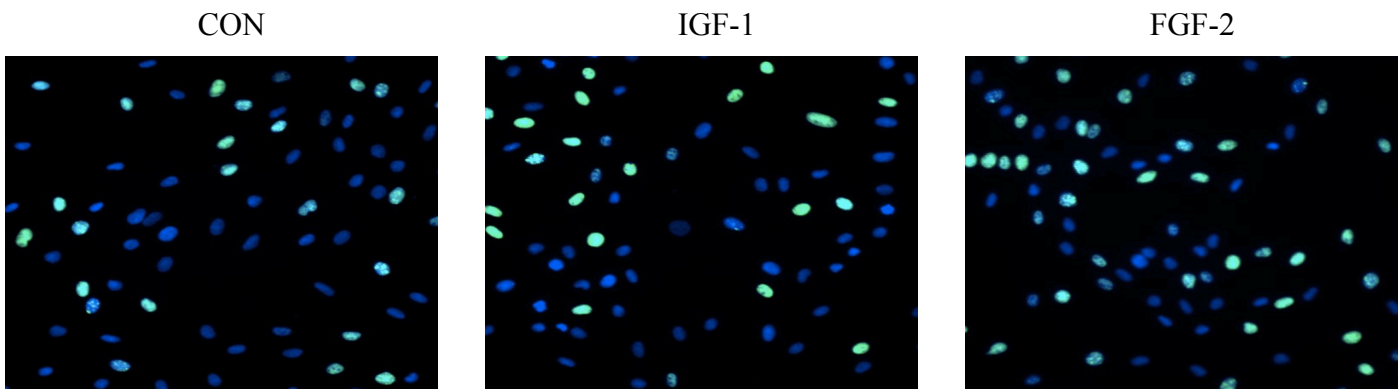
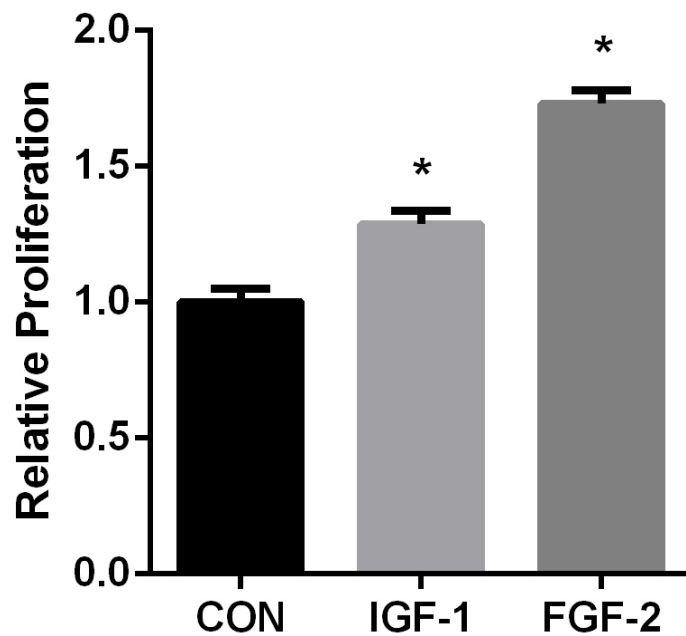
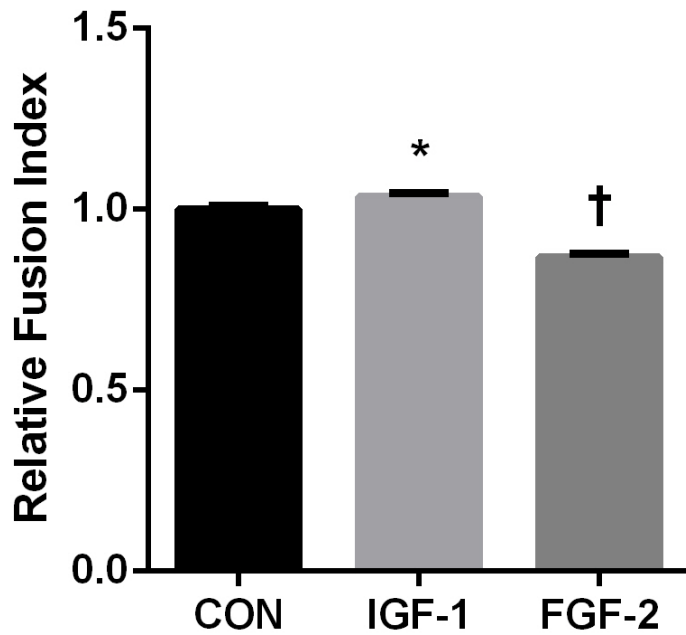


Figure 4. IGF-1 and FGF-2 increase equine satellite cell proliferation. Satellite cells were cultured for 24 h in the presence of 25 ng/mL IGF-1, 10 ng/mL FGF-2 or control media (CON). EdU incorporation was used to determine the number of proliferative cells. Satellite cell proliferation was increased 28.8% in the presence of IGF-1 and 73.0% in the presence of FGF-2 ($P < 0.0001$) compared with controls (CON). Data were normalized to controls and are presented as mean \pm SEM. * $P \leq 0.0001$ compared with CON; $n = 4$ per treatment. Representative images are shown where blue indicates satellite cell nuclei and green indicates proliferative nuclei positive for EdU.



CON

IGF-1

FGF-2

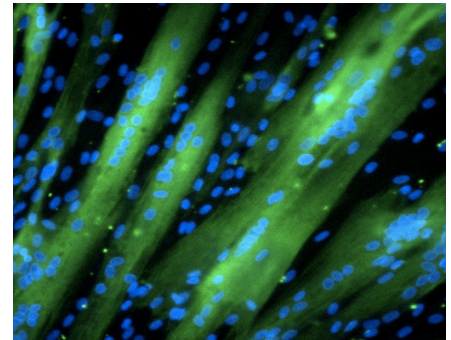
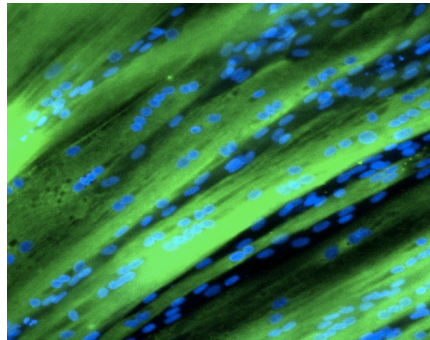
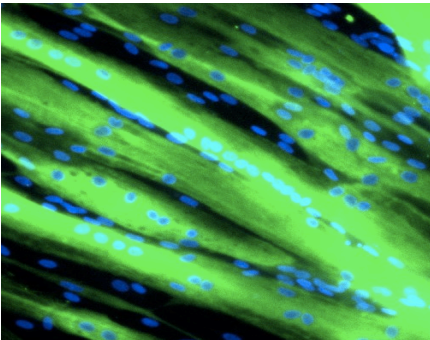


Figure 5. IGF-1 increases equine satellite cell differentiation and FGF-2 decreases equine satellite cell differentiation. Satellite cells were cultured for 48 h in the presence of 25 ng/mL IGF-1, 10 ng/mL FGF-2 or control media (CON). Differentiation was determined by the fusion index. Insulin-like growth factor-1 increased fusion 3.5% ($P = 0.0087$) and FGF-2 decreased fusion 13.1% ($P < 0.0001$) compared with control (CON). Data were normalized to controls and are presented as mean \pm SEM. * $P \leq 0.01$, † $P < 0.0001$ compared with CON; $n = 4$ per treatment. Representative images are shown where blue indicates satellite cell nuclei and green indicates myofibers positive for MyHC.

Discussion

Significant changes occur in equine skeletal muscle as horses develop from weanling to maturity. These changes are evident at the level of the muscle tissue as well as at the cellular and molecular level. Over the course of 1 year, we observed a significant increase in the cross-sectional area of the LD as well as the height, width, and ratio of height to width of the muscle. This growth, however, did not occur at a constant rate. The size of the muscle did not significantly increase during the first six-month period from Oct 13 to Apr 14, but showed a more dramatic increase from Apr 14 to Oct 14. The results suggest that horses undergo more rapid growth during warmer months when they are not expending as much energy for maintenance and thermoregulation. It is logical that horses would not grow as rapidly during a period of reduced energy availability. Brinkmann et al. (2014) found that body mass and body condition score of Shetland ponies on restricted diets during the winter months were significantly decreased compared to summer months and ponies on control diets. The field metabolic rate (FMR) of ponies in both restricted and control groups in the winter was only a third of the FMR measured in the summer. Daily activity as well as heart rate in both restricted and control groups were higher in the summer than in the winter. This study suggests that metabolic activity and energy expenditure is significantly reduced in horses in the winter months. Much of the energy intake goes towards thermoregulation, which could explain why we have observed less growth from Oct to Apr than from Apr to Oct.

Weanlings had a significantly smaller muscle area than horses reaching maturity at 3 to 4 years of age, but horses in the range of 1 to 2 years of age did not have a significantly larger or smaller muscle area than weanlings or 3 to 4-year-olds respectively. The lack of statistically significant differences may have been due to a limited sample size as well as biological

variability between individual horse growth rates and varying breeds. Some horses may grow more rapidly than others due to both genetic and environmental factors. The height and ratio of height to width of the LD showed a very similar trend to the cross-sectional area. The fact that the ratio of height to width increased and there was no significant age effect suggests that the width of the LD does not change to a great extent as horses age. However, this may have been due to image quality of the ultrasound and difficulty determining the outline of the LD.

Treatment with IL-6 decreased proliferation of satellite cells isolated from foals. Because IL-6 is a pleiotropic cytokine, it has the potential to cause different satellite cell responses. It is likely that the relatively high concentration of IL-6 used in this experiment exceeded physiological concentrations appropriate for stimulating activation of satellite cells, and therefore muscle repair. For example, satellite cells isolated from rats increased proliferation in culture in a dose-dependent manner up to 1ng/mL, but treatment with 10ng/mL and 100ng/mL IL-6 did not stimulate proliferation (Kurosaka and Machida, 2013). It can be inferred that at concentrations above 10ng/mL, which was the concentration used in our experiment, IL-6 inhibits proliferation of satellite cells *in vitro*.

Treatment of satellite cells with 20ng/mL TNF- α also caused a significant decrease in proliferation. Although TNF- α is necessary for normal proliferation and differentiation of satellite cells, its effects can differ depending on the physiological state of the individual animal as well as the stage of activation and differentiation (Li, 2003; Podbregar et al., 2013). Tumor necrosis factor- α increased proliferation of satellite cells in a dose-dependent manner from 2 to 6ng/mL, but decreased proliferation at 20ng/mL (Li, 2003). This indicates that TNF- α , like IL-6 can be pleiotropic, but at concentrations exceeding the normal physiological state of the animal,

both cytokines can cause a significant decrease in proliferation of satellite cells, thereby inhibiting muscle repair.

Interestingly, even though proliferation decreased at 10ng/mL IL-6, fusion index increased significantly at this same concentration. In mouse models, IL-6 was critical for increasing fusion of satellite cells into muscle fibers, which supports the results in this experiment (Serrano et al., 2008; Washington et al., 2011). However, differentiation of C2C12 myoblasts was impaired in the presence of 2.5ng/mL (Al-Shanti and Stewart, 2012). The concentration of IL-6 necessary to stimulate differentiation of satellite cells may differ depending on the animal species and may also be different depending on the concentration of IL-6 used in culture. More information on differentiation of equine satellite cells in response to IL-6 is necessary to confirm this.

Differentiation of satellite cells decreased in the presence of 20ng/mL TNF- α . Tumor necrosis factor- α is necessary in moderate concentrations during the inflammation process to stimulate differentiation, however, with prolonged exposure to high concentrations of TNF- α , myoblast differentiation can be inhibited (Magee et al., 2012). In the presence of 20ng/mL TNF- α , myoblast differentiation was decreased *in vitro* (Alter et al., 2008). These findings support the results in our study on equine satellite cells. At what specific concentrations TNF- α stimulates versus inhibits differentiation is still to be determined. Concentrations may differ depending on the age of the animal (Degens, 2010).

Insulin-like growth factor-1 increased both proliferation and differentiation of equine satellite cells. This is consistent with findings in other studies, as well as in other species, given that IGF-1 is a very potent growth stimulator and induces both proliferation and differentiation of

satellite cells and myoblasts (Greene and Allen, 1991; Allen and Boxhorn, 1989). This confirms that IGF-1 acts similarly in young horse muscle as it does in other species studied.

Fibroblast growth factor-2 is a very potent stimulator of proliferation. In this experiment, it increased proliferation by 73.0%, which was the largest change observed throughout the experiment. Satellite cells treated with FGF-2 demonstrated a dose-dependent increase in proliferation (Greene and Allen, 1991; Allen and Boxhorn, 1989). Fibroblast growth factor-2 also induced proliferation in a continuously increasing manner over a period of 96 hours (Byrne et al., 1998). However, FGF-2 significantly decreased differentiation of satellite cells, almost as much as IL-6 (13.1% versus 14.9% respectively). This is consistent with findings in other studies, where FGF-2 decreased differentiation even at relatively low concentrations compared to the 10ng/mL used in this study (Greene and Allen, 1991). It is likely that because FGF-2 is such a potent stimulator of proliferation, that the satellite cells were not able to differentiate because they were consistently being stimulated to proliferate.

Conclusion

In this study, the LD was measured across the span of 1 year in young horses less than 5 years of age. There was an increase in the height, width, cross-sectional area, and height-to-width ratio, particularly observed from Apr 14 to Oct 14. This suggests that the LD undergoes a significant amount of growth in young horses, especially when they are not expending energy over the winter months to conserve heat. Satellite cells are important mediators of muscle growth and can be influenced by a variety of factors. Satellite cell proliferation was decreased in the presence of IL-6 and TNF- α , but increased in the presence of IGF-1 and FGF-2. Satellite cell fusion into myotubes was increased in the presence of IL-6, but decreased in the presence of TNF- α . Fusion was also increased in the presence of IGF-1 and decreased in the presence of

FGF-2. The effects of these cytokines and growth factors may be concentration-dependent, and may also depend on the physiological state of the animal as well as age. The next step in this research is to determine the effects of these growth factors and cytokines on satellite cells isolated from adult and aged horse populations to broaden our understanding of equine muscle physiology and determine if they continue to play a role in maintenance of muscle mass throughout adulthood.

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